PCT/AU03/01265

I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003900236 for a patent by NOVOGEN RESEARCH PTY LTD as filed on 21 January 2003.

WITNESS my hand this
Eighth day of October 2003

J R Yabsley

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Our Ref: 7771040

P/00/009
Regulation 3:2

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s): Novogen Research Pty Ltd
140 Wicks Road
North Ryde New South Wales 2113
Australia

Address for Service: DAVIES COLLISON CAVE
Patent & Trade Mark Attorneys
Level 10, 10 Barrack Street
SYDNEY NSW 2000

Invention Title: **Repair of UV-induced damage in skin**

The invention is described in the following statement:

REPAIR OF UV-INDUCED DAMAGE IN SKIN

Field of the Invention

The present invention relates to the use of equol and dehydroequol in particular, and compounds based on an isoflavonoid ring structure in general to promote repair of DNA damage in skin cells induced by sunlight.

Background

DNA damage in skin cells is particularly important to human health because it can have major effects on skin appearance and well-being, in particular skin carcinogenesis. DNA damage occurs when the ultraviolet (UV) light component (particularly UV-B and UV-C) of sunlight passes through to the lower layers of the epidermis. In its passage through the epidermis, the UV irradiation causes mutations in the DNA strands in the genomes of all cells in the skin. Those mutations are known as pyrimidine dimers which normally are repaired automatically by specialist intra-nuclear enzymes such as endonucleases, with complete repair taking about 2-3 days. Repair involves the excision of the damaged segment and insertion of a new segment. DNA damage caused by UV-induced oxidative stress, which following a complex lengthy cascade resulting in the generation of reactive oxygen species (ROS), takes up to 3 days to occur.

This DNA damage has a number of potentially damaging consequences, particularly where the sunlight exposure is repeated and occurs over many years. These include a small proportion of dimers being mis-repaired, predisposing to mutagenic damage, in particular if the mis-repair occurs in important quality assurance genes such as p53. The accumulation of these mis-repaired genes over a lifetime believed to be a major predisposing factor to skin cancer.

The consequences of UV-induced DNA damage in skin may be associated with photoageing, actinic damage and carcinogenesis. These terms generally have the following meaning:

1. Photoageing refers to the process of accelerated ageing in sunlight-exposed skin. This embraces fine lines and wrinkles, freckles, yellowing of the skin, stretching, dilated capillaries (*telangiectasis*), cherry red spots (*angiomas*), and a dry complexion.

2. Actinic damage refers to pre-malignant or benign skin growths and embraces lesions such as solar keratoses or actinic keratoses.

3. Skin cancer refers to lesions with malignant potential and includes basal cell carcinoma, Bowen's disease (in situ squamous cell carcinoma), squamous cell carcinoma and melanoma.

From this it can be appreciated that any strategy that protects against DNA damage or encourages the repair of DNA damage could be expected to be of considerable benefit to individuals who experience repeated sunlight exposure. To date no such strategy has been described. The main strategy has been to reduce DNA damage by reducing exposure to UV (through the preventive use of sunscreen agents). An alternative strategy is to apply compounds to the skin after sun-exposure that might ameliorate some of the DNA damaging consequences of UV exposure. The use of anti-inflammatory agents, skin rehydration, collagen injections, surgery and dermabrasion are just some of the many cosmetic products and procedures employed in attempts to redress the consequences of photoageing, and actinic damage.

A strategy that was able to promote DNA protection and/or repair would have several important benefits. First, by reducing the time to effect DNA repair, the pathological consequences would be reduced. Second, the repair process would be more efficient with less likelihood of mis-repairs occurring. The benefit of this strategy is confirmed by the use of topical administration of endonucleases in patients with the genetic disorder, xeroderma pigmentosus. Individuals with this condition fail to make endonucleases, the consequence of which is a high risk of malignant skin cancer following sunlight exposure. The application to the skin of these individuals of exogenous endonucleases significantly

reduces the risk of these individuals to skin cancer. Thirdly, by increasing the production of free radical scavengers in the skin, DNA would be protected from oxidative stress lesions that form in response to UV exposure.

It has been speculated that certain compounds, including equol, may have the ability to prevent the onset of some symptoms of ageing in skin (US Patent 6,060,070, Gorbach). The Gorbach patent is concerned with the natural process of ageing that is associated with all tissues in the body and may be associated with reduced estrogen function with advancing age. Lowered collagen content and reduced numbers of elastin fibres in skin as a consequence of falling estrogen levels are though to be the primary factors causing age-related wrinkles. Normal ageing is a distinctive entity to photoageing and normal ageing is not associated with pathological change.

Summary of the Invention

It has now been found by the applicants that compounds of the present invention, namely equol, dehydroequol and other isoflav-3-ene and isoflavan compounds, when applied to the skin or administered orally or parenterally, surprisingly promote repair of pyrimidine dimers and reduce oxidative stress lesions in skin. It was entirely unexpected that the compounds of the present invention promoted DNA repair, and even more surprising to find that they promoted DNA repair and protection.

The application of compounds of the present invention topically to skin following UV-exposure was found to have two important benefits. The first was that the incidence of pyrimidine dimers in skin at 24 hours following UV-exposure was considerably decreased, indicating that these compounds were promoting the rate and extent of DNA repair. The second benefit was that the extent of DNA protection was considerably increased through the increased production of metallothionein. The metallothioneins are a family of proteins produced in response to DNA damage. These are highly effective scavengers of reactive oxygen species (ROS), and in this way reduce the damaging effect of ROS on DNA (Hanada et al).

Detailed Description of the Invention

In accordance with the present invention there is provided use of equol, dehydroequol, or other isoflav-3-ene or isoflavan structures for protecting skin from DNA mutagenic damage associated with UV exposure.

In accordance with another aspect of this invention there is provided use of equol, dehydroequol, or other isoflav-3-ene or isoflavan structures for promoting the rate and extent of DNA repair and protection in skin following UV exposure.

In accordance with another aspect of this invention there is provided use of equol, dehydroequol, or other isoflav-3-ene or isoflavan structures for the prevention and/or treatment of photoageing in skin subject to UV exposure.

In another aspect of this invention there is provided use of the compounds of the invention in the prevention and/or treatment of actinic damage.

In another aspect of this invention there is provided use of compounds of the invention for the prevention and/or treatment of skin carcinogenesis.

In another aspect there is provided use of equol, dehydroequol, isoflav-3-ene or isoflavan compounds for promoting the rate and extent of DNA protection in skin following UV exposure.

In accordance with another aspect of this invention there is provided a method for protecting skin from UV induced DNA mutagenic damage which comprises administering to a subject a composition containing one or more of equol, dehydroequol, or other isoflav-3-ene, or isoflavan compounds in admixture with one or more acceptable carriers and/or excipients.

In accordance with another aspect of this invention there is provided a method for promoting both the rate and extent of DNA repair and protection in skin following UV

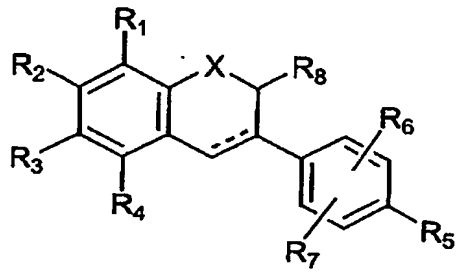
exposure which comprises administering to a subject a composition containing one or more of equol, dehydroequol, or other isoflav-3-ene, or isoflavan compounds in admixture with one or more acceptable carriers and/or excipients.

In accordance with another aspect of this invention there is provided a method for the prevention and/or treatment of photoageing in skin subject to UV exposure which comprises administering to a subject a composition containing one or more of equol, dehydroequol, or other isoflav-3-ene, or isoflavan compounds in admixture with one or more acceptable carriers and/or excipients.

In accordance with another aspect of this invention there is provided a method for the prevention and/or treatment of actinic damage which comprises administering to a subject a composition containing one or more of equol, dehydroequol, or other isoflav-3-ene, or isoflavan compounds in admixture with one or more acceptable carriers and/or excipients.

In accordance with another aspect of this invention there is provided a method for the prevention and/or treatment of skin carcinogenesis which comprises administering to a subject a composition containing one or more of equol, dehydroequol, or other isoflav-3-ene, or isoflavan compounds in admixture with one or more acceptable carriers and/or excipients.

Isoflav-3-ene and isoflavan compounds may be represented by the general formula (II)

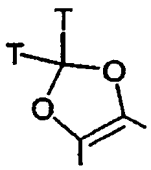


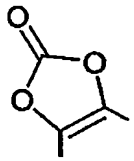
(II)

in which

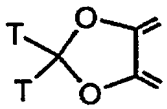
R_1, R_2, R_3 and R_4 are independently hydrogen, hydroxy, OR_9, $OC(O)R_{10}$, $OS(O)R_{10}$, CHO, $C(O)R_{10}$, COOH, CO_2R_{10}, $CONR_{11}R_{12}$, alkyl, haloalkyl, arylalkyl, alkenyl, alkynyl, aryl, heteroaryl, alkylaryl, alkoxyaryl, thio, alkylthio, amino, alkylamino, dialkylamino, nitro or halo, or

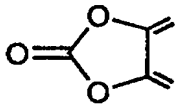
R_3 and R_4 are as previously defined, and R_1 and R_2 taken together with the carbon atoms to which they are attached form a five-membered ring selected from





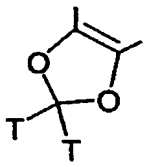
R_1 and R_4 are as previously defined, and R_2 and R_3 taken together with the carbon atoms to which they are attached form a five-membered ring selected from

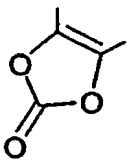




, or

R_1 and R_2 are as previously defined, and R_3 and R_4 taken together with the carbon atoms to which they are attached form a five-membered ring selected from





and

wherein

R_5, R_6 and R_7 are independently hydrogen, hydroxy, OR_9, $OC(O)R_{10}$, $OS(O)R_{10}$, CHO, $C(O)R_{10}$, COOH, CO_2R_{10}, $CONR_{11}R_{12}$, alkyl, haloalkyl, arylalkyl, alkenyl, alkynyl, aryl, heteroaryl, thio, alkylthio, amino, alkylamino, dialkylamino, nitro or halo,

R_8 is hydrogen, hydroxy, alkyl, aryl, amino, thio, $NR_{11}R_{12}$, $CONR_{11}R_{12}$, $C(O)R_{13}$ where R_{13} is hydrogen, alkyl, aryl, arylalkyl or an amino acid, or CO_2R_{14} where R_{14} is hydrogen, alkyl, haloalkyl, aryl or arylalkyl,

R_9 is alkyl, haloalkyl, aryl, arylalkyl, $C(O)R_{13}$ where R_{13} is as previously defined, or $Si(R_{15})_3$ where each R_{15} is independently hydrogen, alkyl or aryl,

R_{10} is hydrogen, alkyl, haloalkyl, amino, aryl, arylalkyl, an amino acid, alkylamino or dialkylamino,

R_{11} is hydrogen, alkyl, arylalkyl, alkenyl, aryl, an amino acid, $C(O)R_{13}$ where R_{13} is as previously defined, or CO_2R_{14} where R_{14} is as previously defined,

R_{12} is hydrogen, alkyl or aryl, or

R_{11} and R_{12} taken together with the nitrogen to which they are attached comprise pyrrolidinyl or piperidinyl,

the drawing "---" represents either a single bond or a double bond, preferably a double bond,

T is independently hydrogen, alkyl or aryl, and

X is O, NR_{12} or S, preferably O,

including pharmaceutically acceptable salts and derivatives thereof.

Preferably compounds of the formula II are equol and dehydroequol.

Most people, including children, teenagers, adults, and the elderly are exposed to UV exposure and sunlight. Indeed, sunlight provides the principal UV exposure experienced by skin. It is believed that most people would benefit from use of compounds of the present invention to promote both the rate and extent of DNA repair in skin following exposure to UV light and sunlight. However, this invention is applicable to any forms of UV exposure, including for example artificial light.

Compounds of the present invention prevent or treat photoageing in skin, actinic damage and/or skin carcinogenesis. Further, compounds of the present invention promote both the rate and extent of DNA repair and protection in skin.

Compounds according to the present invention may be administered topically, orally or parenterally, or by other modes of administration and as mentioned above surprisingly promote repair of DNA pyrimidine, and promote DNA protection through increased production of metallothionein in the skin.

Preferably, compositions containing one or more compounds according to the present invention are applied to the skin either before, at the time of, or after UV or sunlight exposure. For example, compositions may be in the form of a cream, including face cream or skin cream, lotion, cosmetic formulation and the like. For example, compounds of the present invention may be simply mixed, admixed, or blended with suitable carriers or bases to give compositions suitable for application to the skin.

Compounds of the formula II may be generally used in amounts from 20 μg to 500 mg/kg body weight of a subject. Topical compositions may contain compounds of the formula II on a w/w % basis of, for example, 0.01 to 60% w/w, with the remainder comprising carriers and/or excipients and/or standard components used in dermally acceptable compositions as are known in the art.

Compounds of the present invention have preventative and/or treatment applications as described herein. The compounds are preventative in that they lessen, inhibit, or generally prevent DNA damage, and may have the same effects in photoageing in skin subject to UV exposure, actinic damage, and skin cancer. Compounds of the present invention are useful in the treatment of the aforementioned conditions in providing ameliorative outcomes once a subject experiences one or more of the conditions. The compounds of the present invention may be considered as both preventative and as a treatment of the aforementioned conditions in that they may prevent further DNA damage, and may prevent or lessen photoageing, or actinic damage, or skin cancers, whilst at the same time treating the

condition at hand.

In accordance with another aspects of this invention there is provided a method for the treatment, preventing or amelioration of skin cancer, such as basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma, which comprises applying to the skin of a subject a composition containing equol, dehydroequol, or other isoflav-3-ene or isoflavan compounds of the general formula (II).

In another aspect of this invention there is provided a method for increasing metallothionein production in the skin, such as the basal layer of skin, which comprises applying to skin equol, dehydroequol, or other isoflav-3-ene or isoflavan compounds in association with a dermally acceptable carrier.

The applicant has further found that the compounds according to this invention promote DNA repair. The promotion of DNA repair may be by one or more of increasing the rate of repair of cyclobutane pyrimidine dimers (CPDs), promoting DNA repair by decreasing P53 expression, and/or by promoting the formation of metallothionein (MT).

The formation of CPD is considered to be an important lethal and mutagenic consequence of UVR exposure (Mitchell et al, 1989; Liardet et al, 2000). Animal models have demonstrated an inverse relationship between epidermal CPD repair and skin carcinogenesis (Young et al, 1996). The P53 protein (TP53) is expressed after DNA damage by UV irradiation. P53 is a transcription factor which blocks cellular progression from G1 to S phase, thus preventing replication of damaged DNA (Campbell et al, 1993). The P53 protein may act as a tumour promoting agent (Murphey et al, 2001).

This invention will be described with reference to the following, non-limiting examples.

Example 1

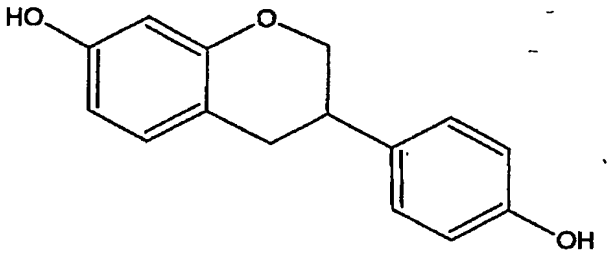
Equol was applied to skin before UV irradiation. Twenty-four hours after UV irradiation, MT production was measured. A control lotion was also used containing no equol. This

experiment demonstrated that equol caused a statistically significant (P=0.469) elevation in the level of MT in the basal layer of irradiated skin (24 hour post-UV) when compared with unirradiated base line skin (pre-UVR). The vehicle itself did not statistically alter the level of MT in the basal layer of irradiated skin, when compared with unirradiated base line skin. Figure 1 herewith shows the results of one experiment.

Example 2

Cyclobutane Pyrimidine Dimers (CPD):

The formation of CPD's, which occurs immediately (Viv Reeve, *pers comm*) would be unaffected by any therapeutic agent applied post-UVR. However, the rate of repair of CPDs might be increased by equol. If this occurred, fewer CPDs in equol treated skin compared with the number in vehicle-only treated skin would be observed.



NV07 or Equol (CAS No. 531-95-3)

There were few CPDs in the unirradiated skin of the volunteer, who demonstrated the expected marked elevation 10 minutes after UV exposure (Table 1). Subject A demonstrated a lower percentage of CPD+ve epidermal cells in equol treated skin.

Table 1: Relative numbers of CPD+ve cells in the epidermis.

	Baseline	0%
	10min	100%
Subject A	Vehicle	52%
	Equol	1%

These results have been presented graphically in Figure 2.

The histological appearance of biopsies from Subject A are shown in Figure 3. The CPD+ve cells are stained a reddish brown. There were few positive cells in the unirradiated baseline biopsy, whereas almost all cells were positive 10 minutes after the UV exposure. There was a marked reduction in the number of CPD+ve cells in the equol treated skin compared with the vehicle treated skin 24 hours after UV exposure. In this volunteer, the results suggest that equol caused an increase of DNA repair.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

References

Campbell, C., Quinn, A.G., Angus, B., Farr, P.M. and Rees, J.L. (1993) "Wavelength specific patterns of p 53 induction in human skin following exposure to UV radiation" *Cancer Research* **52**(12): 2697-9

Hanada, K., Baba, T., Hashimoto, I., Fukui, R. and Watanabe, S (1992) "Possible role of cutaneous metallothionein in protection against photo-oxiditative stress-epidermal localization and scavenging activity for superoxide and hydroxyl radicals" *Photodermatology, Photoimmunology & Photomedicine* **9**(5): 209-13

Liardet, S., Scaletta, C., Panizzon, R., Hohlfeld, P., and Laurent-Applegate L. (2001) "Protection against pyrimidine dimers, p 53, and 8-hydroxy-2'-deoxyguaosine expressionin ultraviolet-irradiated human skin by sunscreens: Difference between UVB + UVA and UVA alone sunscreens" *Journal of Investigative Dermatology* **117**: 1437-1441

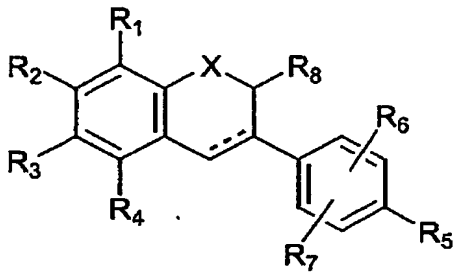
Mitchell, D.L. and Nairn, R.S. (1989) "The biology of the (6-4) photoproduct" *Photochemistry & Photobiology* **49**(6): 805-19

Murphey, R., Young, A.R., Wulf, H.C., Kulms, D. and Schwarz, T. (2001) "The molecular determinants of sunburn cell formation" *Experimental Dermatology* **10**(3): 155-60

Young, A.R., Chadwick, C.A., Harrison, G.I., Hawk, J.J., Nikaido, O. and Potten, C.S. (1996) "The in situ repair kinetics of epidermal thymine dimers and 6-4 photoproducts in human skin types I and II" *Journal of Investigative Dermatology* **106**(6): 1307-13

Claims

1. Use of compounds of the formula II for protecting skin from DNA mutagenic damage associated with UV exposure, wherein said compounds of the formula II comprise



(II)

in which

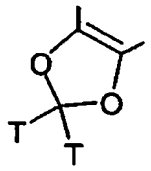
R_1, R_2, R_3 and R_4 are independently hydrogen, hydroxy, OR_9, $OC(O)R_{10}$, $OS(O)R_{10}$, CHO, $C(O)R_{10}$, COOH, CO_2R_{10}, $CONR_{11}R_{12}$, alkyl, haloalkyl, arylalkyl, alkenyl, alkynyl, aryl, heteroaryl, alkylaryl, alkoxyaryl, thio, alkylthio, amino, alkylamino, dialkylamino, nitro or halo, or

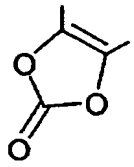
R_3 and R_4 are as previously defined, and R_1 and R_2 taken together with the carbon atoms to which they are attached form a five-membered ring selected from

R_1 and R_4 are as previously defined, and R_2 and R_3 taken together with the carbon atoms to which they are attached form a five-membered ring selected from

, or

R_1 and R_2 are as previously defined, and R_3 and R_4 taken together with the carbon atoms to which they are attached form a five-membered ring selected from





and

wherein

R_5, R_6 and R_7 are independently hydrogen, hydroxy, OR_9, $OC(O)R_{10}$, $OS(O)R_{10}$, CHO, $C(O)R_{10}$, COOH, CO_2R_{10}, $CONR_{11}R_{12}$, alkyl, haloalkyl, arylalkyl, alkenyl, alkynyl, aryl, heteroaryl, thio, alkylthio, amino, alkylamino, dialkylamino, nitro or halo,

R_8 is hydrogen, hydroxy, alkyl, aryl, amino, thio, $NR_{11}R_{12}$, $CONR_{11}R_{12}$, $C(O)R_{13}$ where R_{13} is hydrogen, alkyl, aryl, arylalkyl or an amino acid, or CO_2R_{14} where R_{14} is hydrogen, alkyl, haloalkyl, aryl or arylalkyl,

R_9 is alkyl, haloalkyl, aryl, arylalkyl, $C(O)R_{13}$ where R_{13} is as previously defined, or $Si(R_{15})_3$ where each R_{15} is independently hydrogen, alkyl or aryl,

R_{10} is hydrogen, alkyl, haloalkyl, amino, aryl, arylalkyl, an amino acid, alkylamino or dialkylamino,

R_{11} is hydrogen, alkyl, arylalkyl, alkenyl, aryl, an amino acid, $C(O)R_{13}$ where R_{13} is as previously defined, or CO_2R_{14} where R_{14} is as previously defined,

R_{12} is hydrogen, alkyl or aryl, or

R_{11} and R_{12} taken together with the nitrogen to which they are attached comprise pyrrolidinyl or piperidinyl,

the drawing "---" represents either a single bond or a double bond, preferably a double bond,

T is independently hydrogen, alkyl or aryl, and

X is O, NR_{12} or S, preferably O,

including pharmaceutically acceptable salts and derivatives thereof.

2. Use according to claim 1 for promoting the rate and extent of DNA repair in skin following UV exposure.

4. Use according to claim 1 for the prevention and/or treatment of photoageing in skin subject to UV exposure.

5. Use according to claim 1 for the prevention and/or treatment of actinic damage.

6. Use according to claim for the prevention and/or treatment of skin carcinogenesis.

7. Use according to claim 1 involving over expression of metallothioneins in the skin, particuarly the basal layer of skin.

8. A method for protecting skin from UV induced DNA mutagenic damage which comprises administering to a subject a composition containing one or more of equol, dehydroequol, or other isoflav-3-ene, or isoflavan compounds in admixture with one or more acceptable carriers and/or excipients.

9. A method according to claim 8 which is a method for promoting the rate and extent of DNA repair and protection in skin following UV exposure.

10. A method according to claim 8 which is a method for the prevention and/or treatment of photoageing in skin subject to UV exposure.

11. A method according to claim 8 which is a method for the prevention and/or treatment of actinic damage.

12. A method according to claim 8 which is a method for the prevention and/or treatment of skin carcinogenesis.

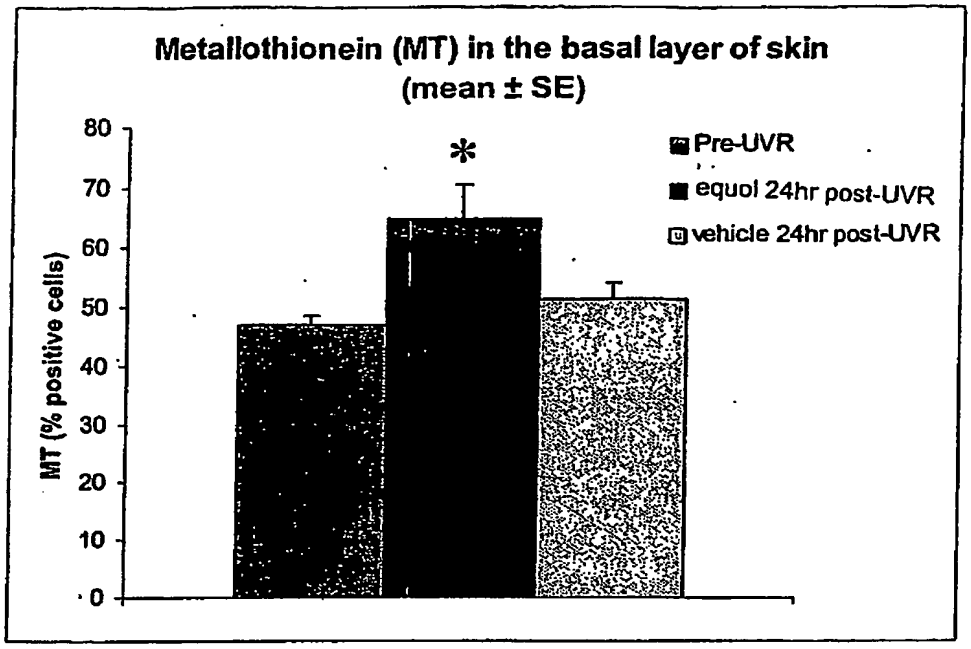
13. Use according to any of claims 2 to 7 wherein compounds of the formula II comprise equol and dehydroequol.

14. A method according to any of claims 8 to 11 wherein compounds of the formula II comprise equol and dehydroequol.

DATED this 21st day of January, 2003.

Novogen Research Pty Ltd
By Its Patent Attorneys
DAVIES COLLISON CAVE

FIGURE 1



Equol caused a statistically significant (*p + 0.0469) elevation in the level of MT in the basal layer of irradiated skin (24hr post-UV), when compared with unirradiated baseline skin (pre-UVR), whereas vehicle did not statistically alter the level of MT in the basal layer of irradiated skin, when compared with unirradiated baseline skin (p = 0.3971)

FIGURE 2

Percentage of CPD+ve cells in the epidermis

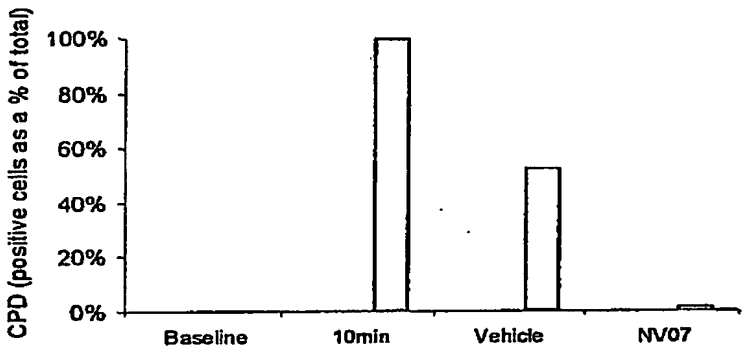


FIGURE 3

Skin biopsies stained with the mAB H3 which detects CPD (samples from subject A)